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Running Title: Tschimganidine Alleviates Metabolic Diseases

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ABSTRACT

Obesity increases the risk of mortality and morbidity because it results in hypertension, heart disease, and type 2 diabetes. Therefore, there is an urgent need for pharmacotherapeutic drugs to treat obesity. We performed a screening assay using natural products with anti-adipogenic properties in 3T3-L1 cells and determined that tschimganidine, a terpenoid from the Umbelliferae family, inhibited adipogenesis. To evaluate the anti-obesity effects of tschimganidine *in vivo*. Mice were fed either a normal chow diet (NFD) or a high-fat chow diet (HFD) with or without tschimganidine for 12 weeks. Treatment with tschimganidine decreased lipid accumulation and adipogenesis, accompanied by reduced expression of adipogenesis and lipid accumulation-related factors. Tschimganidine significantly increased the phosphorylation of AMP-activated protein kinase (AMPK) and decreased that of AKT. Depletion of AMPK relieved the reduction in lipid accumulation resulting from tschimganidine treatment. Moreover, tschimganidine administration drastically reduced the weight and size of both gonadal white adipose tissue (WAT) and blood glucose levels in high-fat diet-induced obese mice. We suggest that tschimganidine is a potent anti-obesity agent, which impedes adipogenesis and improves glucose homeostasis. Tschimganidine can then be evaluated for clinical application as a therapeutic agent.

INTRODUCTION

The rise in obesity and obesity-related metabolic diseases is an epidemic worldwide (1) . In the United States, over 78 million adults are obese and have comorbidities (2). Obesity is characterized by chronic low-grade inflammation of adipose tissue. Obesity also influences several physiological processes, such as gut permeability, gastric emptying, cardiac output, and liver and renal function (3). Therefore, the development of therapeutic agents to prevent and manage obesity and obesity-related metabolic diseases is important.

Previously, we performed a screening analysis to identify natural compounds with anti-obesity effects (4). We identified tschimganidine, a terpenoid, to exhibit the expected effect of inhibiting lipid accumulation in 3T3-L1 cells. Its structure is shown in Fig. 1A. Tschimganidine is a member of the Umbelliferae family (5). Terpenoids have been reported to be selectively toxic against gram-positive bacteria (6) and to have anti-cancer effects (7). However, there are only two reports on the biological activity of tschimganidine, i.e., it can act as an agonist of oestrogen receptor-alpha (ERα) (8) and extend the lifespan of yeast (9). Tschimganidine can also act as a phytoestrogen (10). Phytoestrogens are natural phytochemicals that have a function similar to gonadal oestrogen hormones and are potential alternatives to hormone replacement therapy (5). Among phytoestrogens, terpenoids can exhibit both oestrogenic and anti-oestrogenic activities by targeting oestrogen receptors (11). In particular, tschimganidine, by functioning as an ERα agonist has oestrogen-mimicking characteristics (8). ERα agonists have been reported to exert anti-obesity effects by stimulating oestrogen receptors (12). However, research on these effects of terpenoids is lacking, so, it is worthwhile to conduct a study on how tschimganidine affects obesity.

Based on these points, we first hypothesized that tschimganidine, as an ERα agonist, would have a therapeutic effect on metabolic diseases such as obesity and fatty liver disease. And we aimed to elucidate the molecular mechanism by which tschimganidine reduces lipid accumulation in adipocytes. Herein, we investigated the effect of tschimganidine on adipocytes and a high-fat diet-obese mouse model to explore whether tschimganidine has potential as a therapeutic agent for obesity in the future.

RESULTS

Tschimganidine reduces lipid accumulation in 3T3-L1 cells without cytotoxicity

The 3T3-L1 preadipocytes were treated with different concentrations of tschimganidine two days after the induction of cell differentiation to evaluate the effect of tschimganidine. Adipogenesis, the process by which adipocyte precursors develop into mature adipocytes, was measured by ORO staining of the lipid droplets on day 6 (Fig. 1B). ORO staining showed that tschimganidine reduced lipid accumulation dose-dependently. Tschimganidine treatment at 5 μg/mL showed a reducing effect on lipid accumulation. The tschimganidine doses which were most effective for inhibiting adipocyte differentiation and lipid accumulation were 25 and 50 μg/mL, respectively (Fig. 1B). A cell viability assay was conducted to evaluate whether the lipid accumulation inhibitory effect of tschimganidine resulted from cell cytotoxicity. We confirmed that there was no significant difference in cell viability between tschimganidine-treated and control cells (Fig. 1C). The expression of adipogenesis-related proteins, namely PPARγ, and its downstream factors, C/EBPα, FABP4, and FASN, decreased after tschimganidine treatment dose-dependently (Fig. 1D). Tschimganidine (15 μg/mL) was observed via ORO staining to have a dose-dependent inhibitory effect on lipid accumulation (Fig. 1E). These results suggest that tschimganidine inhibits adipogenic differentiation by downregulating the expression of adipogenesis-associated proteins.

Tschimganidine reduces the expression levels of adipogenesis-related factors after adipocyte differentiation

Whole cell lysates were obtained on days 0, 2, 4, and 6 to examine how tschimganidine treatment affects adipogenesis and lipid accumulation-related genes during adipocyte differentiation. The 3T3-L1 cells were treated with tschimganidine two days after adipogenic stimulation. Tschimganidine significantly suppressed the mRNA (Fig. 2A) and protein expression (Fig. 2B) of PPARγ, C/EBPα, FABP4, and FASN after two days of treatment. We further investigated whether the inhibitory effect of tschimganidine occurs at the early or late stages of adipogenesis. Cells were treated with

methylisobutylxanthine-dexamethasone-insulin (MDI) and tschimganidine on day 0. Tschimganidine treatment reduced adipocyte differentiation and lipid accumulation (Supplementary Fig. 1). The effects of tschimganidine on late adipocyte differentiation were also examined. The 3T3-L1 cells were incubated with tschimganidine from days 6 to 10. Tschimganidine treatment reduced lipid accumulation and lipid droplet size in 3T3-L1 cells compared to control cells. The ORO staining results showed that tschimganidine retarded all stages of adipocyte differentiation, including the early stage (days 0 to 2) and the late stage (days 6 to 10). These data indicate that tschimganidine represses adipogenesis and reduces lipid accumulation in the adipocytes.

Tschimganidine induces phosphorylation of AMP-activated kinase (AMPK) and Acetyl-CoA carboxylase (ACC)

Cell signalling molecules, such as AKT, AMPK, ERK, and JAK2, participate in adipogenesis and lipid accumulation (18). To investigate the molecular mechanisms of tschimganidine on adipogenesis and lipid accumulation, we examined the changes in the activation of these signalling pathways from day 2 to 3 of adipocyte differentiation (Fig. 2C). The expression levels of AKT, AMPK, ERK 1/2, and JAK2 were unaffected by tschimganidine treatment. However, levels of phosphorylated AKT, ERK 1/2, and JAK2 were significantly decreased after tschimganidine treatment. Tschimganidine also increased AMPK phosphorylation. These results showed that tschimganidine reduced the phosphorylation of adipogenesis-related signalling pathways during the early stages of adipocyte differentiation. We also found that tschimganidine treatment increased AMPK and ACC phosphorylation time-dependently (Fig. 2D). Multiple studies have shown that AMPK is activated by phosphorylation and inhibits adipocyte differentiation (19-21).

We examined whether tschimganidine inhibits lipid accumulation through AMPK activation. The suppression effect of tschimganidine on lipid accumulation was reduced by the knockdown of AMPK gene expression using AMPK siRNAs. ORO staining, lipid droplet size, and lipid accumulation in tschimganidine-treated 3T3-L1 cells after AMPK-knockdown were lower than those of the control

cells (Fig. 2E). Moreover, AMPK knockdown reduced the inhibitory effect of tschimganidine on the expression of genes related to adipogenesis and lipid accumulation (Fig. 2F). Therefore, tschimganidine inhibits adipogenesis and lipid accumulation via AMPK activation.

Tschimganidine affects body weight, insulin tolerance, and glucose tolerance in high-fat diet-fed mice

The anti-obesity effects of tschimganidine were evaluated *in vivo*. Mice were fed an NFD or HFD (containing 60% kcal as fat) for 12 weeks, and intraperitoneal injection of tschimganidine was started from 5 weeks after the HFD. Tschimganidine treatment reduced body size in HFD-fed obese mice, whereas there was no difference in body size in NFD-fed mice, regardless of tschimganidine treatment (Fig. 3A). The reduction in body weight was notable in HFD-fed mice (Fig. 3B). In particular, mice treated with 5 μg/kg tschimganidine had a lower body weight than the control mice. However, there were no significant differences in food intake between the two diet groups (Fig. 3C).

Tschimganidine effects on metabolic parameters, such as alanine aminotransferase (ALT), glucose, and triglyceride (TG) levels, were evaluated in the serum of experimental mice. Tschimganidine reduced the levels of ALT, glucose, and TG in the HFD-fed mice (Fig. 3D). We also tested whether tschimganidine improves glucose homeostasis *in vivo*. Tschimganidine treatment significantly improved glucose tolerance compared to control conditions in HFD-fed mice (Fig. 3E). Insulin sensitivity was analysed using the ITT. There were no differences in blood glucose levels between the tschimganidine-treated and the untreated control NFD-fed mice; however, the blood glucose level was lower in the tschimganidine-treated HFD-fed mice than in the control HFD-fed mice (Fig. 3F). These data indicate that tschimganidine delays the increase in body weight and ameliorates metabolic parameters such as ALT, blood glucose, and TG levels, insulin tolerance, and glucose tolerance in HFD-fed mice.

Tschimganidine reduces adipocyte size and adipogenesis-related factors in the fat tissues of HFD-fed mice

The amount of gWAT and iWAT in NFD- and HFD-fed mice with or without tschimganidine treatment was evaluated. The amounts of gWAT and iWAT were reduced in tschimganidine-treated HFD-induced obese mice (Fig. 4A). Tschimganidine-treated mice from the HFD-fed group had lower gWAT and iWAT weights than those from the control group (Fig. 4B). H&E staining of paraffin-embedded gWAT was performed for histological analysis. Significantly more reduced-size adipocytes in gWAT were observed in tschimganidine-treated NFD-fed and HFD-fed mice (Fig. 4C). Similarly, tschimganidine-treated mice of both NFD-fed and HFD-fed groups exhibited smaller adipocytes in the iWAT compared to the untreated control mice (Fig. 4D).

The mRNA expression of FABP4, PPARγ, C/EBPα, and FASN, markers of adipogenesis and lipid accumulation in gWAT and iWAT, were evaluated. The mRNA expression levels of FABP4, PPARγ, C/EBPα, and FASN decreased in the gWAT of the tschimganidine-treated NFD-fed and HFD-fed mice (Fig. 4E). Similarly, tschimganidine inhibited the mRNA expression of FABP4, PPARγ, C/EBPα, and FASN in the iWAT of HFD-fed mice (Fig. 4F). Tschimganidine also decreased C/EBPα and FASN levels in the iWAT of NFD-fed mice. Tschimganidine treatment also diminished adipocyte size in the brown adipose tissue of both NFD-fed and HFD-fed mice, as revealed by histological analysis (Supplementary Fig. 2). Taken together, tschimganidine reduced the weight and cell size of both gWAT and iWAT by downregulating adipogenesis-associated gene expression.

Tschimganidine reduces steatosis in the liver tissues of high-fat diet-fed mice

We determined whether tschimganidine inhibits lipid accumulation in the liver tissues of NFD-fed- and HFD-fed mice. The liver size was reduced in tschimganidine-treated HFD-fed obese mice (Supplementary Fig. 3A). The liver weight of tschimganidine-treated mice was also lower than that of control mice in the HFD-fed group (Supplementary Fig. 3B). Next, we conducted H&E staining of paraffin-embedded liver tissues. Lipid accumulation in liver tissues was significantly reduced in tschimganidine-treated HFD-fed mice (Supplementary Fig. 3C). Hepatic TG and free fatty acid (FFA) levels were measured and found to be significantly lower in the liver of tschimganidine-treated mice

than in the control mice (Supplementary Fig. 3D, E). Additionally, mRNA levels of lipid accumulation-related factors, such as FABP4, PPARγ, and FASN, were decreased in the liver tissue of tschimganidine-treated NFD-fed mice (Supplementary Fig. 3F). Similar to that in NFD-fed mice, tschimganidine also repressed the gene expression of FABP4, PPARγ, and FASN in the livers of HFD-fed mice (Supplementary Fig. 3G). Taken together, tschimganidine alleviated hepatic steatosis on HFD-fed mice.

DISCUSSION

Natural compound-derived therapeutics consist of bioactive phytochemicals with beneficial health effects and may be beneficial in treating obesity and metabolic diseases (5). These therapeutics are based on substances extracted from natural products, such as animals, plants, and minerals; therefore, they are popular as sources of medicine and alternative treatments for prescription drugs because of the experience of using natural products for a long time and the expectations for their effectiveness, despite controversy over their safety (22-24). In fact, about 25% of medicines sold today are derived from plants (23). Natural products have several key advantages; they are biologically active and are composed of proteins selected by nature for specific biological interactions (25). Natural products can be derived from secondary metabolites produced to aid the survival of organisms; thus, they can exhibit low mammalian toxicity (26). Herbal secondary metabolites have also been found to contain phytochemicals with beneficial therapeutic effects, such as alkaloids, terpenoids, and phenolics (23). Therefore, they are used in various ways to prepare nutritional supplements, dietary supplements, and genetically modified foods (5). Additionally, several natural product-based drugs have been developed to enhance human health against cancer, cardiovascular disease, and osteoporosis (5).

We hypothesized that tschimganidine has potential as a natural product-derived therapy in the future by inhibiting adipogenesis and lipid accumulation and conducted research on this. In this study, we aimed to uncover the molecular mechanisms by which tschimganidine regulates adipogenesis. First, we determined that treatment with tschimganidine drastically reduced the expression of adipogenesis-

associated genes, such as PPARγ, C/EBPα, FASN, and FABP4, as well as adipogenesis in 3T3-L1 cells. These results were confirmed using tschimganidine at a concentration that maintains cell viability, and it was proposed that the anti-adipogenic effect of tschimganidine is due to the reduced expression of these genes. We determined the optimal concentration of tschimganidine to suppress adipogenesis. Cells treated with 15 μg/mL tschimganidine displayed inhibited differentiation and reduced lipid accumulation based on ORO staining. We confirmed that the expression of adipogenesis-associated genes was significantly reduced. We also observed this effect *in vivo*; tschimganidine administration reduced obesity in mice fed a high-fat diet. The expression of lipid accumulation- and adipogenesis-associated genes, such as FABP4, PPARγ, C/EBPα, and FASN, was significantly decreased in the WATs of these mice. Here, we hypothesised that tschimganidine, a phytoestrogen, inhibits the expression of adipogenesis-associated genes.

We further detected a significant increase in the phosphorylation of AMPK following tschimganidine treatment. AMPK is a heterotrimeric enzyme that plays a major role in maintaining energy homeostasis in various organs and tissues (27). In adipose tissue, it has been reported to play an important role in regulating adipocyte glucose and lipid metabolism (28). When AMPK is activated, it induces the phosphorylation of downstream signalling targets either directly or indirectly. This induction decreases the synthesis of cholesterol and fatty acids (29). Regulation of lipid metabolism is a well-known function of AMPK. AMPK also acts as a cellular sensor of glycogen, which regulates the rate of synthesis and breakdown of this fuel source (30). AMPK is activated by stresses that cause Adenosine triphosphate (ATP) depletion, leading to increased binding of Adenosine monophosphate (AMP) and Adenosine diphosphate (ADP) to specific regulatory sites of this kinase. This results from ATP hydrolysis, which is rapidly converted to AMP by the adenylate kinase reaction (28). It has been reported that activation of ERα induces AMPK phosphorylation, which increases beiging in white adipocytes (31). Therefore, we investigated whether tschimganidine induces AMPK phosphorylation and found that activation of AMPK by tschimganidine increases adipocyte lipolysis, despite the debate regarding whether AMPK could play a role in regulating lipolysis (32, 33). Treatment with tschimganidine

increased phosphorylation of AMPK, accompanied by phosphorylation of ACC. However, the effect of tschimganidine treatment on the inhibition of lipid accumulation was diminished by the knockdown of AMPK gene expression. This indicates that the effect of tschimganidine on lipid accumulation was based on the activation of AMPK, which is the predominant mechanism by which tschimganidine affects adipogenesis and lipid accumulation as an ERα agonist.

Furthermore, Tschimganidine also improved glucose tolerance and insulin resistance in HFD-fed mice. Our data support that tschimganidine increases AMPK activation and induces blood glucose uptake and lipolysis in adipose tissues. Lipid contents were significantly reduced in the liver tissue of the HFD-fed mice. Tschimganidine treatment reduced TG and free fatty acid levels in the liver and decreased the expression of lipid accumulation-associated genes in liver tissues, suggesting that tschimganidine may be beneficial in treating fatty liver and related metabolic diseases. The results of this study show that tschimganidine may act as an effective therapeutic agent for obesity and metabolic diseases by inhibiting adipogenesis through the activation of AMPK. However, further research is needed to determine the detailed mechanism which proteins tschimganidine targets and how this modulates AMPK activity.

In summary, tschimganidine, a terpenoid from the Umbelliferae family, reduces adipogenesis, lipid accumulation, and blood glucose levels through the activation of AMPK. Tschimganidine is a potential therapeutic agent for obesity and metabolic diseases, and further mechanistic studies are needed to evaluate its clinical application.

MATERIALS AND METHODS

Materials and methods are described in Supplementary Information.

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CONFLICTS OF INTEREST

All authors have no conflicts to declare.

FIGURE LEGENDS

Fig. 1. Tschimganidine reduces lipid accumulation in 3T3-L1 cells without cytotoxicity (A) Structure of tschimganidine. (B) ORO staining of tschimganidine-treated 3T3-L1 cells. After MDI induction, 3T3-L1 cells were treated with tschimganidine on days 2 to 6. ORO staining was performed on day 6. Measurement of lipid accumulation. ORO was eluted with 100% isopropanol from the stained cells, and the absorbances were measured at 500 nm. ***P <0.001, dimethyl sulfoxide vs. tschimganidine. (C) Cell viability assays. Confluent 3T3-L1 cells were treated with tschimganidine for 48 h. (D) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein expression was normalized to that of β-actin. (E) ORO staining of tschimganidine-treated 3T3-L1 cells. After MDI induction, 3T3-L1 cells were treated with tschimganidine on days 2 to 6. ORO staining was performed on day 6.

Fig. 2. Tschimganidine reduces the expression levels of adipogenesis-related factors after adipocyte differentiation through activation of AMPK. (A) mRNA expression of PPARγ, C/EBPα, FABP4, and FASN was detected using real-time polymerase chain reaction. RNA samples were prepared on days 0, 2, 4, and 6. 3T3-L1 cells were treated with tschimganidine on days 2 to 6. mRNA expression was normalized to that of β-actin. *P <0.05, **P <0.01, ***P <0.001, DMSO vs. tschimganidine. (B) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein samples were prepared on days 0, 2, 4, and 6. 3T3-L1 cells were treated with tschimganidine on days 2 to 6. Protein expression was normalized to that of β-actin. (C) Western blotting of signal transduction-related proteins. 3T3-L1 cells were treated with tschimganidine on day 2 and incubated for 24 h. (D) The activity of signal transduction-related proteins, such as AMPK and ACC, was detected by western blotting. 3T3-L1 cells were treated with tschimganidine on day 2. (E) ORO staining and lipid accumulation of AMPKa1 knockdown-3T3-L1 cells with or without tschimganidine. After transfection of AMPKa1 small interfering RNA (siRNA), 3T3-L1 cells were differentiated using MDI. Then, 3T3-L1 cells were

treated with tschimganidine on day 2. ORO staining was performed on day 6. ***P <0.001, scRNA vs. siAMPK. (F) Protein expression of PPARγ, C/EBPα, FABP4, and FASN was detected by western blotting. Protein samples were prepared on day 4. Protein expression was normalized to that of β-actin.

Fig. 3. Tschimganidine reduces body weight gain and improves insulin and glucose tolerances in high-fat diet-fed mice. (A, B) Decreased body size and weight of HFD-fed mice treated with tschimganidine for 12 weeks. Tschimganidine was administered to both HFD-fed and NFD-fed mice. Body weight was recorded every two days. *P < 0.05, **P < 0.01; (n = 6 per each group) (C) Food intake was measured by weighing the remaining chow. (D) Measurement of ALT, glucose, and triglyceride levels. Blood was drawn and analysed from the NFD- or HFD-fed mice with or without tschimganidine treatment. (E, F) Glucose tolerance test and insulin tolerance test of. Vehicle (DMSO) or tschimganidine (1 or 5 μg/kg) treated mice. Blood glucose levels were measured at 15, 30, 60, 90, and 120 min. *P <0.05, **P < 0.01; (n = 6 per each group of NFD-fed mice and n = 5 per each group of HFD-fed mice). vehicle vs. tschimganidine 5 μg/kg.

Fig. 4. Tschimganidine reduces adipocyte size and adipogenesis-related factors in fat tissues of high-fat diet-fed mice. (A) White adipose tissues of NFD- or HFD-fed mice administered vehicle (DMSO) or tschimganidine. (B) Gonadal and inguinal WAT weight was measured after the 12-week diet period. (C) The adipocyte size in gonadal WAT sections was determined by staining with H&E. Size measurements were performed using ImageJ software. (D) The adipocyte size of inguinal WAT sections was determined by staining with H&E, and the size measurements were performed as above. (E, F) Gonadal and inguinal WAT were collected, and tissue lysates were prepared. The mRNA expression of FABP4, PPARγ, C/EBPα, and FASN was detected by real-time PCR analysis. β-actin was used as a normalization control. Data are presented as the mean ± SD; *P < 0.05; **P < 0.01; and ***P < 0.001 for vehicle vs. 1 and 5 μg/kg tschimganidine treatment.

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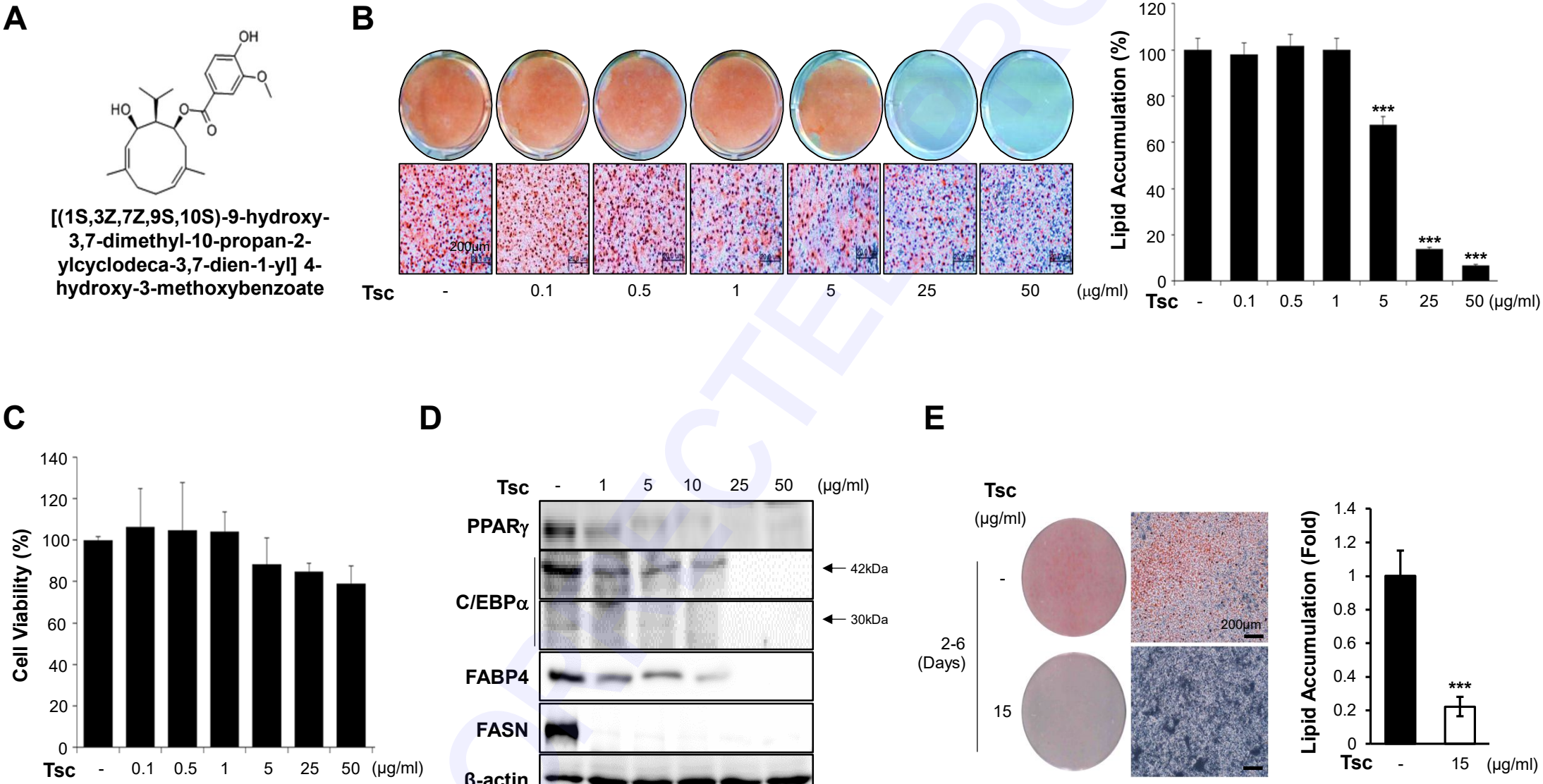
Figure 1


A
[(1S,3Z,7Z,9S,10S)-9-hydroxy-3,7-dimethyl-10-propan-2-ylcyclodeca-3,7-dien-1-yl] 4-hydroxy-3-methoxybenzoate
B
200μm
Tsc - 0.1 0.5 1 5 25 50 (μg/ml)
Lipid Accumulation (%)
120
100
80
60
40
20
0
***
***
***
Tsc - 0.1 0.5 1 5 25 50 (μg/ml)
C
Cell Viability (%)
140
120
100
80
60
40
20
0
Tsc - 0.1 0.5 1 5 25 50 (μg/ml)
D
Tsc - 1 5 10 25 50 (μg/ml)
PPARγ
C/EBPα
42kDa
30kDa
FABP4
FASN
β-actin
E
Tsc
(μg/ml)
-
2-6
(Days)
15
200μm
Lipid Accumulation (Fold)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
***
Tsc - 15 (μg/ml)

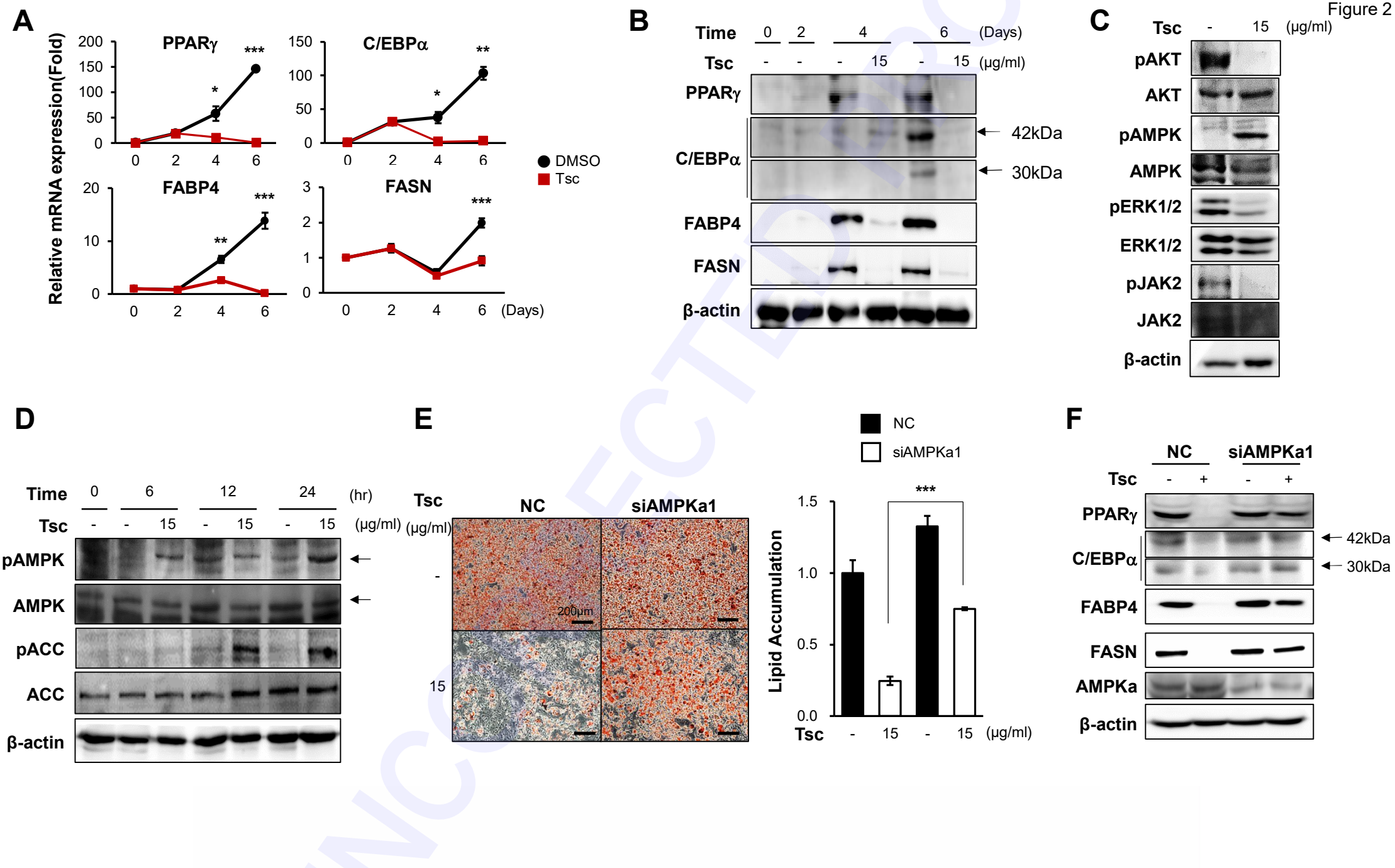

A
Relative mRNA expression(Fold)
PPARγ
C/EBPα
FABP4
FASN
DMSO
Tsc
(Days)
B
Time
Tsc
(Days)
(μg/ml)
PPARγ
C/EBPα
42kDa
30kDa
FABP4
FASN
β-actin
C
Tsc
(μg/ml)
pAKT
AKT
pAMPK
AMPK
pERK1/2
ERK1/2
pJAK2
JAK2
β-actin
D
Time
(hr)
Tsc
(μg/ml)
pAMPK
AMPK
pACC
ACC
β-actin
E
Tsc
(μg/ml)
NC
siAMPKa1
200μm
Lipid Accumulation
F
NC
siAMPKa1
Tsc
PPARγ
C/EBPα
42kDa
30kDa
FABP4
FASN
AMPKa
β-actin

Figure 3

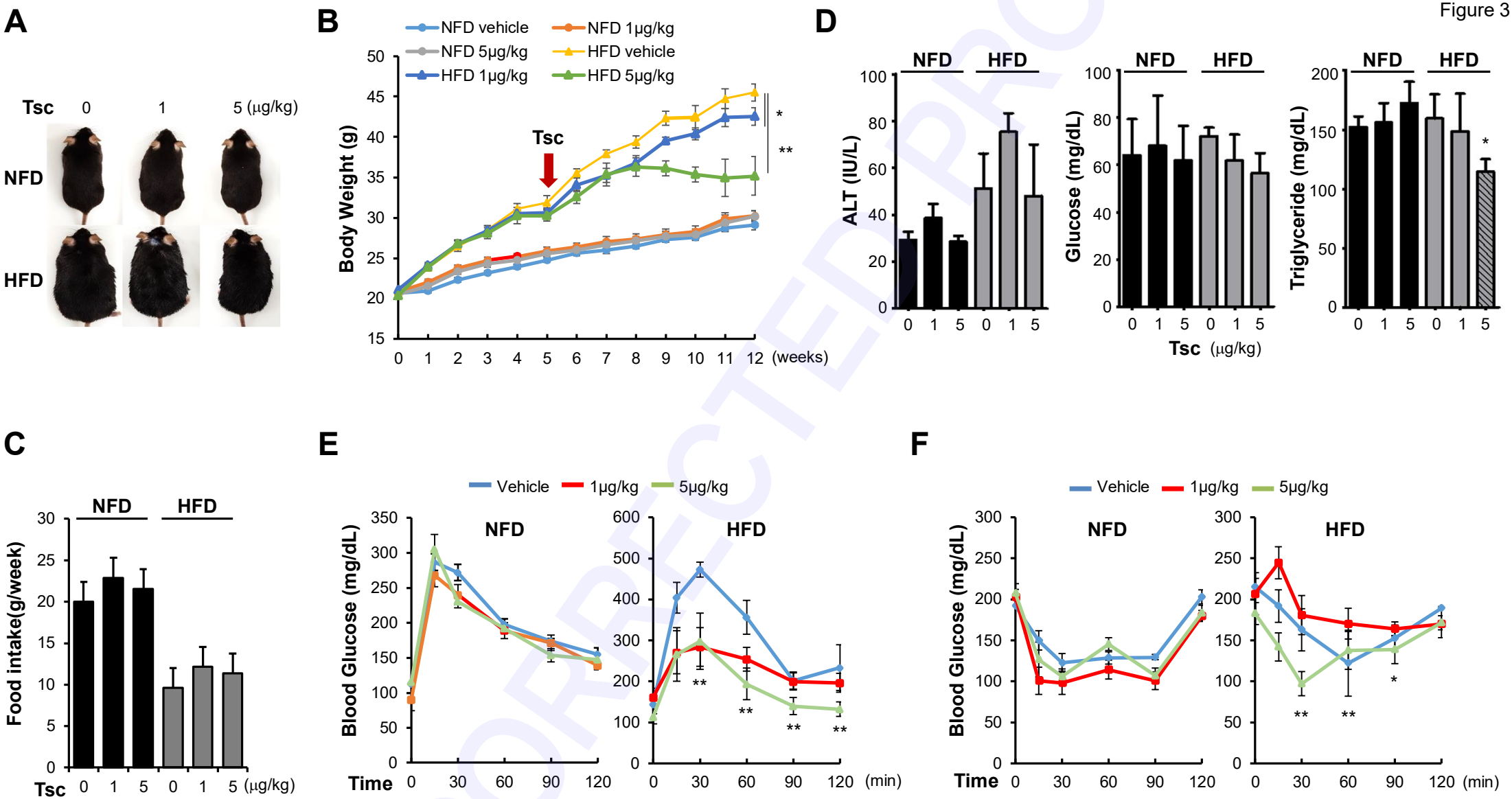
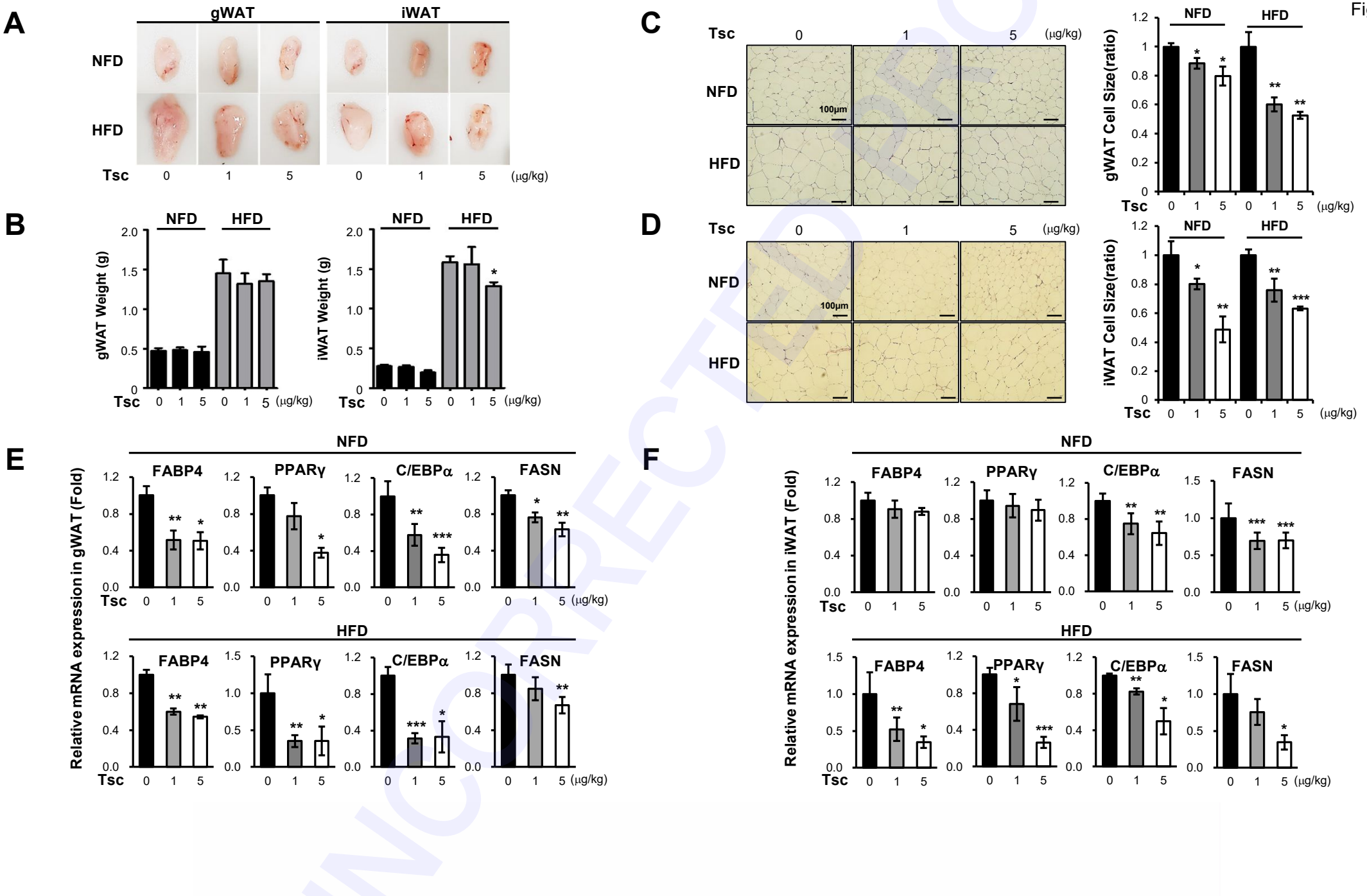


Figure 4


A
gWAT
iWAT
NFD
HFD
Tsc 0 1 5 0 1 5 (μg/kg)
B
NFD HFD
gWAT Weight (g)
iWAT Weight (g)
Tsc 0 1 5 0 1 5 (μg/kg)
C
Tsc 0 1 5 (μg/kg)
NFD
HFD
100μm
gWAT Cell Size(ratio)
D
iWAT Cell Size(ratio)
E
NFD
HFD
FABP4
PPARγ
C/EBPα
FASN
Relative mRNA expression in gWAT (Fold)
F
Relative mRNA expression in iWAT (Fold)

Supplementary Information

MATERIALS AND METHODS

Chemicals

Natural product library was purchased from Enzo Life Sciences. Tschimganidine, isobutylmethylxanthine (IBMX), dexamethasone, insulin, and ORO powder were purchased from Sigma Chemicals.

Cell culture and adipocyte differentiation assay

The 3T3-L1 cells were maintained and differentiated as previously described (4, 13). The 3T3-L1 cells were grown to a post-confluence state using Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum. On day 0 post-confluence, 3T3-L1 cells were incubated on DMEM supplemented with 10% foetal bovine serum (FBS), insulin (1 μg/mL), IBMX (520 μM), and dexamethasone (1 μM). After 2 days, the medium was replaced with DMEM supplemented with 10% FBS and insulin (1 μg/mL). After 4 days, the medium was replaced with DMEM supplemented with 10% FBS.

Cell viability assay

The 3T3-L1 cells were cultured in 12-well plates and incubated until they reached confluence. Cells were then treated with tschimganidine for 48 h at the indicated concentrations. Cell viability was measured using Ez-Cytox (Daeil Lab Co. Ltd.), according to the manufacturer's protocol.

Oil Red O staining

Differentiated 3T3-L1 cells were incubated in 10% formalin for 10 min and washed with distilled water. The cells were then stained with ORO in 60% isopropanol. The stain retained by the cells was eluted with 100% isopropanol, and the OD_{500} of the solution was measured with microplate reader (Molecular devices).

Western blotting analysis

Cell lysate extractions were prepared using Radio-immunoprecipitation assay (RIPA) buffer (1% Triton X-100; 1% sodium deoxycholate; 0.1% sodium dodecyl sulfate; 150 mM NaCl; 50 mM Tris-HCl, pH 7.5; and 2 mM EDTA, pH 8.0) as previously described(13, 14). Primary antibodies (antibodies against C/EBPα, PPARγ, FABP4, FASN, p-AMPK, AMPK, p-AKT, AKT, p-JAK2, and JAK2 from Santa Cruz Biotechnology; antibodies against p-ACC, ACC, p-ERK1/2, and ERK1/2 from Cell Signalling) were used for detection using a FUSION SOLO S (Vilber) detector, according to the manufacturer's instructions. The normalisation control used was anti-β-actin (Santa Cruz Biotechnology).

RNA isolation and real-time PCR analysis

Total mRNA was isolated from tissue samples and cultured cells using an RNA lysis reagent (easy-BLUE™; iNtRON Biotechnology) according to the manufacturer's instructions. cDNA (1 μg) was synthesised from RNA using qPCR RT Master Mix (Takara Bio). The following primers were used for real-time PCR amplification: PPARγ: F_5′-AGGGCGATCTTGACAGGAAA-3′ and R_5′-CGAAACTGGCACCCTTGAAA-3′; C/EBPα: F_5′-GACATCAGCGCCTACATCGA-3′ and R_5′-TCGGCTGTGCTGGAAGAG-3′; FABP4: F_5′-CATCAGCGTAAATGGGGATT-3′ and R_5′-TCGACTTTCCATCCCACTTC-3′; FASN: F_5′-TGGGTTCTAGCCAGCAGAGT-3′ and R_5′-ACCACCAGAGACCGTTATGC-3′; and β-actin: F_5′-GGCTGTATTCCCCTCCATCG-3′ and R_5′-CCAGTTGGTAACAATGCCATGT-3′. Real-time PCR was performed using SYBR Green Master Mix (Toyobo) and an Applied Biosystems thermocycler.

Transfection of small interfering RNA

The 3T3-L1 cells were cultured in a 6-well plate and incubated for 24 h. The cells were transfected with mouse AMP-activated protein kinase catalytic subunit alpha-1 (AMPKa1) siRNA at 50 nM (Bionics) using Lipofectamine™ RNAiMAX (Invitrogen) according to the manufacturer's protocol (15, 16). After 24 h, the medium was replaced with DMEM supplemented with 10% bovine serum. The next day,

cell differentiation was induced by transferring the cells to DMEM supplemented with 10% FBS, insulin (1 μg/mL), isobutylmethylxanthine (520 μM), and dexamethasone (1 μM).

Mice and diets

Wild-type C57BL6/J male mice were purchased from Orient Bio. Treatment included 1 and 5 μg/kg of tschimganidine injected intraperitoneally. To assess metabolic parameters, eight-week-old mice were fed either a normal chow diet (NFD) or a high-fat chow diet (HFD, 60% of kcal as fat, Research Diets) with or without tschimganidine for 12 weeks. Body weight and food intake were measured every 2 days to evaluated the effect of tschimganidine treatment. All mouse tissues were frozen in liquid nitrogen and stored at -80 °C until the experiments were performed. All animal experiments were approved by the Institutional Review Board of the Yonsei University College of Medicine. All mice were housed in pathogen-free facilities, in a 12 hours' light/dark cycle in ventilated cages, with chow and water supply ad libitum according to the university's guidelines for the Care and Use of Laboratory Animals (permission number for animal experiment: 2015-0094).

Analysis of serum metabolic parameters

Blood was collected from the mice mentioned above and centrifuged for 10 min at 132 × *g* to obtain serum. Serum analysis including alanine aminotransferase, glucose and Triglyceride was performed at the Seoul Medical Science Institute.

Metabolic assays

Glucose tolerance tests (GTTs) were performed by intraperitoneally injecting mice with 1 g/kg glucose (Sigma-Aldrich); insulin tolerance tests (ITTs) were performed by intraperitoneally injecting mice with 1 U/kg insulin (humulin). Blood glucose levels were measured 0, 15, 30, 60, 90, and 120 min after injection using a glucometer.

Morphological analysis of tissues

Gonadal white adipose tissue (gWAT), inguinal white adipose tissue (iWAT), liver, and brown adipose tissue (BAT) were fixed in 4% paraformaldehyde for 24–48 h at 4 °C, processed for paraffin embedding, and stained with haematoxylin and eosin (H&E). The cell size was analysed using ImageJ software (17).

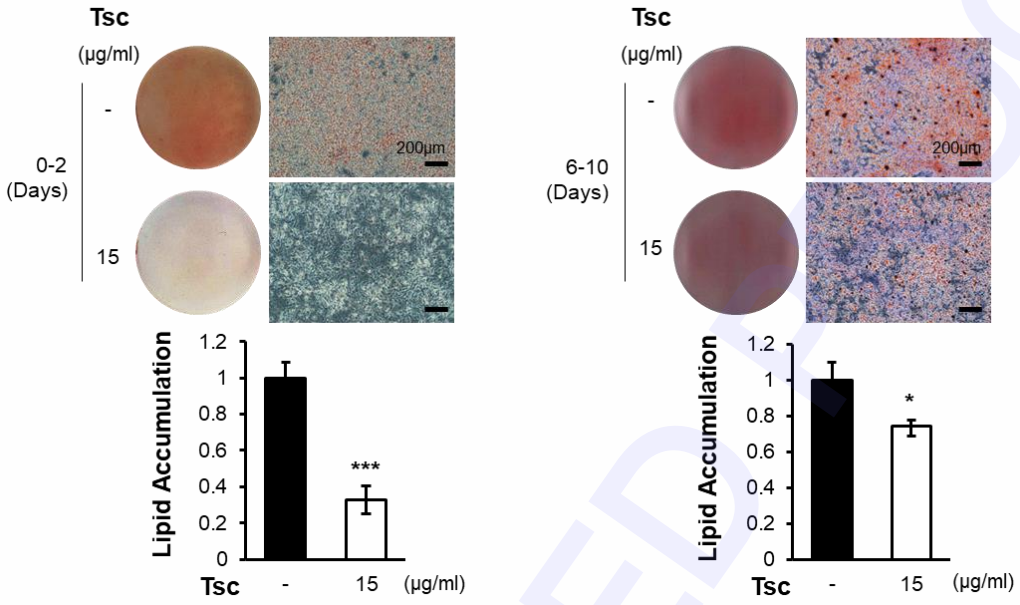
Quantification of hepatic triglycerides and free fatty acids

Liver triglycerides and Free fatty acids content were measured in lipid extracts from previously frozen liver tissues of NFD or HFD mice with or without tschimganidine for 12 weeks using the Triglyceride Quantification Colorimetric Kit (BioVision), the Free fatty acid Quantification Colorimetric Kit (K612-100; BioVision), following the manufacturer's instructions. Colorimetric measurements were performed at 570 nm using microplate reader (Molecular devices). All experiments were measured at least three times in triplicate.

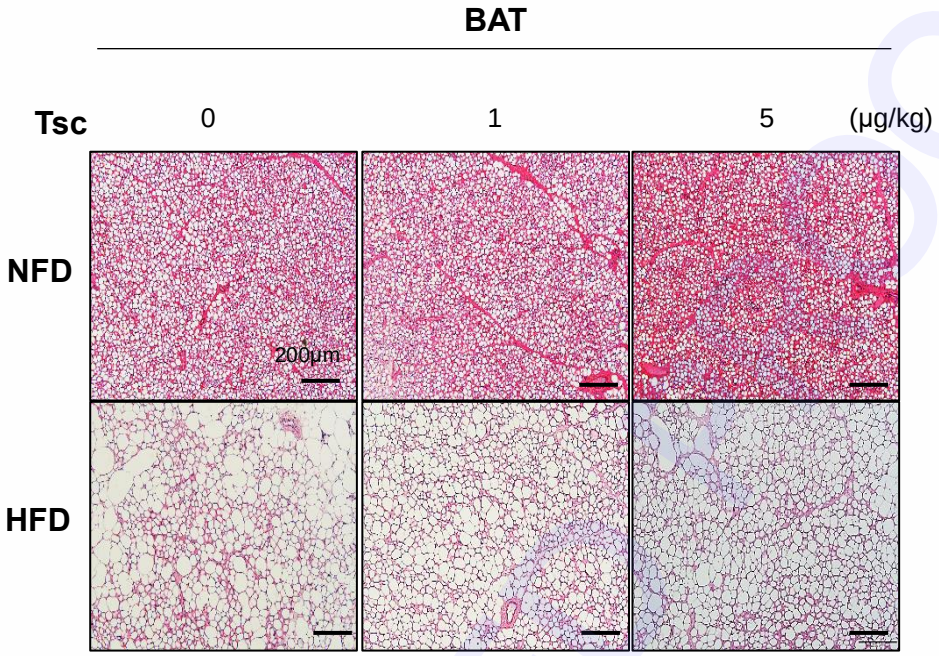
Statistical analysis

Unpaired *t*-tests were used to compare two groups. Statistical analyses were performed using Prism 5 (Graph-Pad). Statistical significance was set at $P < 0.05$.

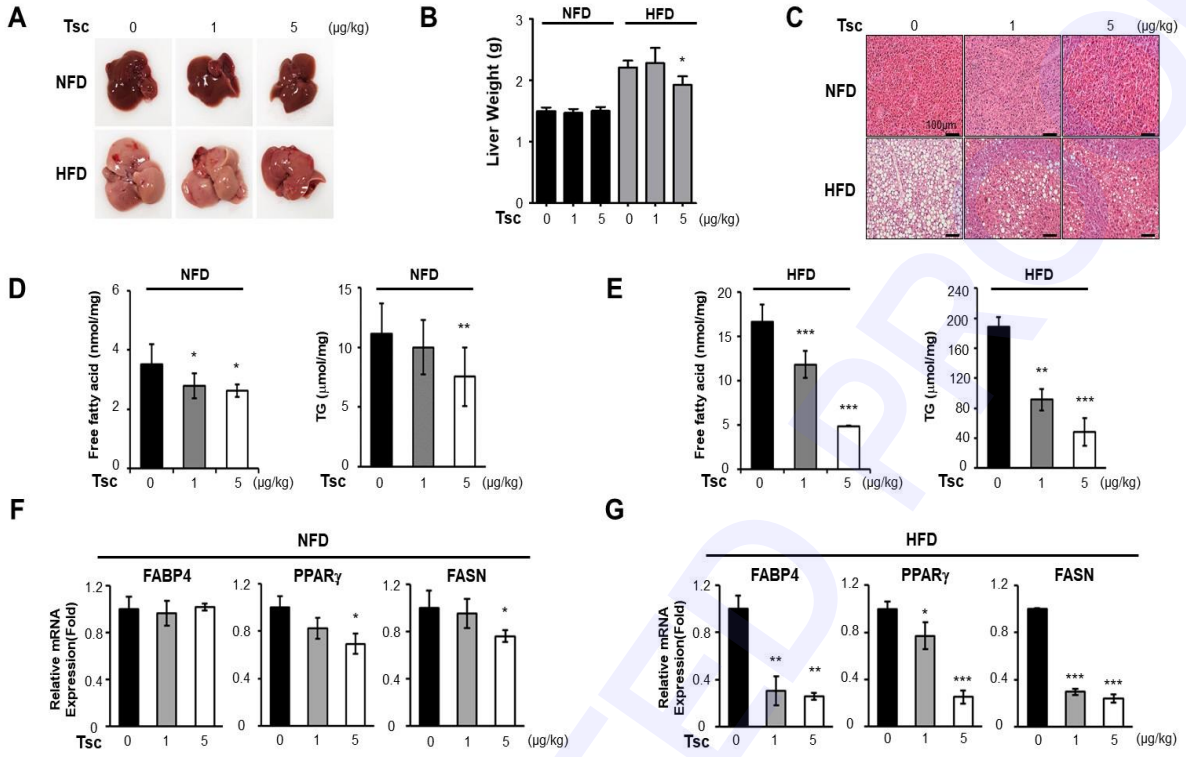
Supplementary figure



Supplementary Fig. 1. The effect of tschimganidine on all stages of adipocyte differentiation ORO staining and lipid accumulation of tschimganidine-treated 3T3-L1 cells. 3T3-L1 cells were treated with MDI and tschimganidine for day 2 (left panel). ORO staining was performed on day six. 3T3-L1 cells were treated with tschimganidine on days 6 to 10 (right panel). ORO staining was performed on day 10. ORO, Oil Red O; MDI, methylisobutylxanthine-dexamethasone-insulin.



Supplementary Fig. S2. The effect of tschimganidine on the brown Adipose Tissues. H&E staining of BAT sections was performed in each group treated with or without Tschimganidine (Tsc) for 10 weeks to observe lipid content. BAT, brown adipose tissue; NFD, normal fat diet; HFD, high-fat diet; H&E, hematoxylin and eosin. Scale bar: 200 μm.



Supplementary Fig. 3. Tschimganidine reduces liver weight and adipogenesis-related factors in liver tissues of high-fat diet-fed mice. (A, B) The size and weight of liver tissue from NFD- or HFD-fed mice. Liver weight was measured after the 12-week diet period. (C) H&E staining of liver sections was performed in each group treated with or without tschimganidine for 10 weeks. (D) Hepatic TG and FFA levels. TG and FFA from the liver were extracted, and their levels were measured using the colorimetric method at 570 nm. (E, F) Liver tissue lysates were prepared, and the mRNA expression of FABP4, PPARγ, and FASN was detected by real-time PCR. β-actin was used as a normalization control. Data are presented as the mean ± SD; $*P < 0.05$; $**P < 0.01$; and $***P < 0.001$ for vehicle vs. 1 and 5 μg/kg tschimganidine treatment. NFD, normal fat diet; HFD, high-fat diet; FFA; free fatty acid, TG, triglyceride; PCR, polymerase chain reaction.